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Original Article

SCREENING OF PHARMACOLOGICAL AND CYTOTOXIC ACTIVITIES OF FRESH WATER LAKE ISOLATED MICROALGAE CHLORELLA VULGARIS AS-13 AND CHLORELLA PYRENOIDOSA AS-6

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ABSTRACT

Micro Algae are rich sources of biologically active metabolites. Pharmaceutical industries nowadays are focusing on isolation and extraction of primary and secondary metabolites of medicinal importance produced by these organisms. The present investigation is to evaluate phenolic and flavonoid content in methanolic extracts of freshwater algal biomass isolated from Unkal Lake, Dharwad district, Karnataka, India, followed by evaluation of antioxidant and anticancer activities. Determination of total phenol and flavonoid content was followed by the 2,2-diphenyl-1-picrylhydrazyl radicals scavenging, phosphomolybdate and reducing power assay for evaluating antioxidant potency. Among two microalgae, Chlorella vulgaris AS-13 had efficiently high amount of total phenol and flavonoid content. At 1000 µg/ml concentration of extract, Chlorella vulgaris AS-13 and Chlorella pyrenoidosa AS-6 exhibited 105 and 88 % diphenyl-1-picryhydrazyl free radical scavenging activity and 78 and 61% total antioxidant potency respectively. The cytotoxicity of Methanol extract of Chlorella vulgaris AS-13 showed 50% inhibition (IC50) of cells with 92 µg/ml after 72 hours period incubation for MCF7 when compared to HepG2 cell lines. Whereas there was a positive effect of methanolic extract of Chlorella pyrenoidosa AS-6 on MCF7 at 72 hour period of with 87 µg/mL. Thus, both of the microalgae in dietary supplements will be effective mediators in scavenging free radicals and act as antiapoptotic peptides for tumor cells.

KEYWORDS: Microalgae, Phenols, Flavonoids, Antioxidant Activity & Anticancer Activity

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INTRODUCTION

Cancers are the cause of genetic mutations in the DNA of the cancerous cells and these mutations are inheritance mutation, metabolic mutations, radiations, excessive cigarette smoking, alcohol consumption and chemical pollutants. All these causative agents or elements act individually or act in sequence or together to pledge or stimulate cancers [1, 2, 3]. Chemotherapy is usually the first line treatment to cure most cancers. Besides that, a group of drugs are used to kill or inhibit the growth of cancer cells. These drugs are allied through noxiousness that ranges from a mild reaction to severe life-threatening illness. Many side effects of chemotherapeutic drugs comprised baldness, vomiting, Canker sores, diarrhea, loss of appetite, nausea and fatigue. Hence new anticancer agents should be investigated from various resources. A lot of natural antitumor amalgams or their by products are generally produced by blue-green algae [4]. Remarkable and exhilarating biological activities are displayed by microalgae like Immune suppressant, antitumor, antimicrobial and antiviral activities which are conspicuous targets

of biomedical investigations. Bioactive molecules with anticancer activities are one major group of targeted compounds from microalgae. Hence, there is a need to extract natural bioactive amalgams from algae [5]. Compounds obtained from algae have assumed a vital part in the improvement of a few clinically valuable anticancer managers. In most cases, the evaluation of the anticancer potential of crude extracts from different sea organisms has been carried out by *in vivo* cytotoxicity tests in malignant cell cultures. The capacity of algal polysaccharides to incite cancer cell multiplication has been overall recorded. The epidemiological information is upheld by rat model studies exhibiting defensive impacts of dietary kelps and other red and green algae against mammary tumors [6]. Molecular and cellular level studies on algae have indicated that algae-derived bioactive are potent cancer inhibitors. Documentation of new active cancer inhibiting agents has globally turn out to be a significant strategy [7]. A variety of red algae as well as kelp has anti-proliferative and anti-inflammatory activities. *In vivo* and *in vitro* studies on seaweed constituents have been conducted to explicate the anti-mutagenic mechanisms of underlying the potential anti-carcinogenic effects of kelp and red algae against colon and breast cancers [8, 9].

MATERIALS AND METHODS

Algae isolation and Culturing

Microalgae samples were isolated from the Unkal lake, Dharwad District, Karnataka, India [19] a freshwater lake. 250 ml of water sample was filtered through the membrane with pore size of 0.22 μm using Buckner funnel with 0.2 vacuum. After filtration, the membrane was washed in 5 ml of distilled autoclaved water. 30 μl of the above 5 ml solution was inoculated on 100 μl of BG-11 medium and incubated at 25 ^{o}C ± 2 ^{o}C under a light intensity of 56 μmol m^{-2} s^{-1}. After 15 days, cultures were serially diluted and plated for axenic culture.

Morphological Characterization of Microalgae

Morphology of microalgal cells was done through light microscopy by simple wet mount method. The aliquot of each sample was placed in the center of the clean glass slide and covered with a thin cover slips. The microalgae were examined under light a microscope (Karl Zess) by 100X objective lens and were photographed. Surface morphology of isolated Microalgae was analyzed through SEM microscopy. The sample was initially fixed with glutaraldehyde and postfixed with Osmium tetraoxide. It was then dehydrated with ethanol and allowed to dry completely and was examined under the scanning electron microscope (JSM-6390LV, Jeol, Japan).

Preparation of Algal Extracts

Isolated algal cultures, were maintained at 24±1°C in a thermostatically controlled room and illuminated with cool inflorescence lamps (Oreva 40 W, cool daylight 6500 K) at an intensity of 2000 lux in a 12:12 hr light and dark cycle. At log phase, algal biomass was harvested. About 100 gram of the collected fresh algal biomass of each culture equal to 10 g of dried one was completely homogenized and extracted with 250 ml of methanol solvent. Clarification of algal mixture was carried out by filtration method using Whatman No.1 filter paper. The clarified extracts were evaporated under a vacuum at 50 °C using a rotary evaporator. The crude extracts were stored in the dark in a vial and kept at 4 °C until further analysis.

Total Phenols and Flavonoids

In total phenol content determination, standard procedure was followed using Folin-Ciocalteu's reagent and sample absorbance was measured at 725 nm [11]. Gallic acid was used as a standard for a calibration curve; the total phenol was expressed in gallic acid equivalents. The total flavonoid was determined by measuring the sample absorbance at 415 nm [12]. Quercetin was used as a standard for a calibration curve; the total flavonoid content was expressed in quercetin equivalents. Each of the above assays using algal extracts was performed in triplicate.

Radical Scavenging Assay (DPPH)

The free radical scavenging activity of different fractions was measured *in vitro* by using 0.1 mM solution of 2, 2-diphenyl-1-picrylhydrazyl DPPH in 95% methanol [13]. To 1 ml of this solution, 1 ml of each fraction dissolved in methanol at different concentrations were added and allowed to stand at room temperature for 30 min in dark. Then absorbance was measured at 517 nm against methanol blank and percentage of scavenging inhibitions were determined by comparing with ascorbic acid as standard. Percentage inhibition (I%)=(Ac–As)/Ac×100, where, Ac is the absorbance of the control and As is the absorbance of the sample.

Reducing Power Assay

The reducing power of different fractions of methanol extracts (1.0 ml) was assayed by the addition of 2.5 ml of phosphate buffer (50 mM, pH 7.0) and 2.5 ml of 1% potassium ferricyanide [15]. After incubation of reaction mixtures at 50 °C for 20 min, 2.5 ml of trichloroacetic acid (10%) was added to the mixtures and centrifuged at 3000 rpm for 10 min. From the supernatant, 1.25 ml was mixed with 1.25 ml of distilled water and 0.25 ml FeCl3 solution (0.1% w/v) [10]. Finally, the absorbance was measured at 700 nm against methanol blank; increased absorbance values indicate higher reducing power.

In Vitro Cytotoxic Assay

Confluent cell cultures were harvested with a trypsin-EDTA solution and plated onto 96-well plate at the cell density of approximately 1 X 104 cells / well. Serial dilution of a sample was carried out on the plate with the highest concentration of microalgae extract being 100 µg/mL. Each test well was added with 100 µL of the diluted microalgae extract. Later 100 µL of cells to be tested were added to the wells making up the volume to a total of 200 µL of solution. The plates were then incubated at 37 ^{o}C in the CO_2 incubator. The assay was carried out with different exposure times which were 24, 48 and 72 hours. At the end of the incubation period, 20 µL of MTT solution was added to each test well. The plate was later incubated for 3 to 4 hours to allow the reaction to take place. Following incubation, most of the solution in each well was discarded leaving the purple formazan precipitate at the bottom of the well. Then, 100 µL of DMSO was added to each well and the solution was pipetted thoroughly to dissolve the purple formazan crystals. The amount of formazan produced after treatment was read using a microplate ELISA reader at the wavelength of 570 nm. The absorbance was recorded. The IC50 values (concentration of tested compound required to inhibit cell proliferation by 50%) were determined from the dose-response inhibition curve.

RESULTS AND DISCUSSIONS

Isolated Microalgae were examined with a light microscope 100 × magnification and it was found that they are unicellular, size varied from 3-5 micron and the chloroplast was cup-shaped (Figure 1). Surface morphology of isolates

was studied by Scanning Electron Microscopy at 10,000 × Magnification, the isolated microalgae were morphologically identified as *Chlorella vulgaris* and *Chlorella pyrenoidosa*. Isolation and enumeration of algae were done as per the methods described by Adoni *et al.,* (1985), Agarker, *et al.,* (1994), Welch, (1948), Hosmani and Bharathi, (1980). Identification was done by consulting the monographs by Philipose (1967), Gandhi (1998) and Prescott (1998). *Chlorella vulgaris* AS-13 is single cell size of 6 µm × 4 µm and *Chlorella pyrenoidosa* AS-6 is also a single cell organism with a cell size of 5.89 µm × 3.45 µm (Figure 2).

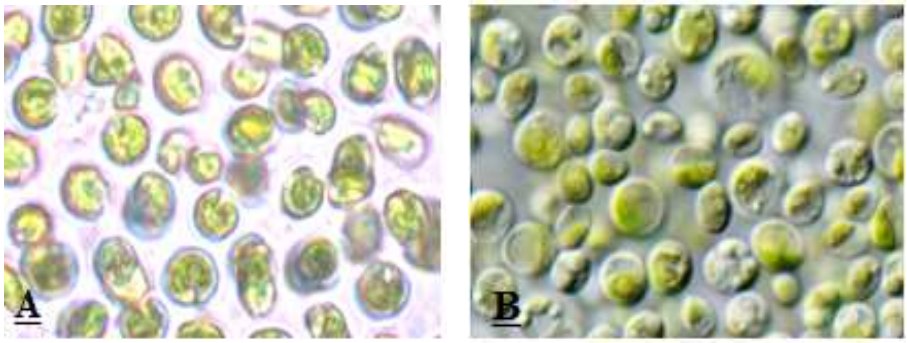

Figure 1: **Light Microscopy at 100 × Magnification Image of Isolates (a) Chlorella Vulgaris AS-13 and (b) Chlorella Pyrenoidosa AS-6**

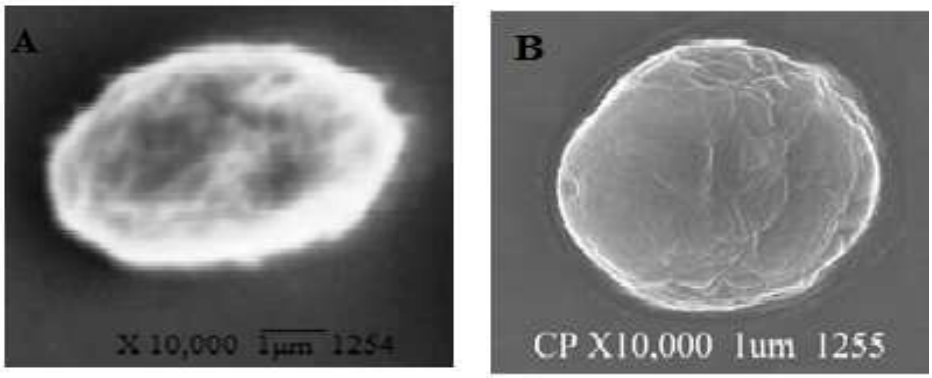

Figure 2: Scanning Electron Microscopy at 10,000 × Magnification Image of Isolates (a) *Chlorella Vulgaris* AS-13 and (b) *Chlorella Pyrenoidosa* AS-6

Phenolic compounds are commonly found in plants, encompassing seaweeds, and have been reported to have a wide range of biological activities including antioxidant and anticancer activities. Phenolic compounds act as antioxidants by chelating metal ions, preventing radical formation and improving the antioxidant endogenous system [18]. The present study revealed that the amounts of polyphenols and flavonoids in tested algae as well as their anticancer activity. Results showed that the amount of total phenolic and total flavonoid contents in the Methanolic extracts of *Chlorella vulgaris* AS-13 and *Chlorella pyrenoidosa* AS-6 were 700.18 ± 25 mg/g and 500.33 ± 25 mg/g dry weight expressed as gallic acid equivalents and 324.12 ± 25 mg/g and 335.59 ± 25 mg/g, expressed as rutin equivalents, respectively (Table 1); these levels were significantly higher than those reported for seaweeds [14]. Similar results were obtained by Bhaskar *et al.,* 2004, who tested the phenol and flavanoid contents in seaweeds of marine coasts of Tamil Nadu.

Table 1: Total Polyphenol and Flavonoid contents of Methanolic Extracts of the Testedalgae

	Polyphenols Content * (mg GAE/g DW)	Flavonoids Content (mg Rutin/g DW)
Chlorella vulgaris AS-13	700.18 ± 25 mg/g	324.12 ± 25 mg/g
Chlorella pyrenoidosa AS-6	500.33 ± 25 mg/g	335.59 ± 25 mg/g

*Results were recorded as (mean ± SD); *mg GAE/g DW: milligram gallic acid equivalent per gram dry weight; mg Rutin /g DW: milligram Rutin equivalent per gram dry weight. Each value is presented as mean ± SD (n = 3). Means within each column with different letters (a-f) differ significantly (P < 0.05).*

Free radical scavenging ability of methanol extract of *Chlorella vulgaris* AS-13 was maximum of 105.56% at 1000 µg/ ml with IC50 value of 412.51 µg/ml; *Chlorella pyrenoidosa* AS-6 was maximum of 88.68% at 1000 µg/ml with IC50 value of 443.34 µg/ml and standard (ascorbic acid) of 94.08 % at 1000 µg/ml with IC50 value of 127.52 µg/ ml (Table 2). The phosphomolybdenum assay is based on the reduction of Mo (VI) to Mo (V) in the presence of antioxidants resulting in the formation of a green phosphate/Mo (V) complex exhibiting maximum absorption at 695 nm. Based on reduction ability, radical scavenging activity of methanol extract of *Chlorella vulgaris* AS-13 was maximum of 92.90% at 1000 µg/ml with an IC50 value of 54.78 µg/ml; *Chlorella pyrenoidosa* AS-6 was maximum of 82.74% at 1000 µg/ml with an IC50 value of 73.06 µg/ ml and standard same as above, respectively (Table 2).

Table 2: DPPH Assay and Total Antioxidant Assay

Conc^{n} (µg/ml)	DPPH Assay		Phosphomolybdate Assay		Standard (Ascorbic Acid)
	C. vulgaris AS-13	*C. pyrenoidosa* AS-6	*C. vulgaris* AS-13	*C. pyrenoidosa* AS-6	
62	18.17±0.19	7.25±0.08	56.58±1.32	42.43±0.69	23.82±0.56
125	21.53±0.27	22.49±0.32	78.13±1.82	61.48±1.90	49.01±1.14
250	37.01±0.63	28.67±0.89	89.33±2.08	75.82±1.00	64.54±1.50
500	62.97±1.47	49.04±0.52	92.25±2.15	79.97±2.34	78.60±1.83
1000	105.56±2.6	88.68±0.23	97.90±2.28	82.74±1.32	94.08±2.20
IC50 (µg/ml)	412.51	443.34	54.78	73.06	127.52

Results are expressed as the average of triplicate determination ± standard deviation

Reducing capacity is considered as a significant additional indicator of the potential antioxidant activity of a compound or sample [21]. Reducing the power of *C. vulgaris* AS-13 extract increased to 0.268 at 1000 µg/ml, in *C. pyrenoidosa* AS-6 extract increased to 0.189 at 1000 µg/ml whereas standard had the absorbance of 0.710 at 1000 µg/ml (Table 3); the reducing power of the extracts increased in dose-dependent manner. The studies conducted by Rabia Alghazeer *et al.*, 2016, showed that the alcoholic extracts of the brown algae *C. crinite*, *C. stricta* and *S. vulgare* possessed good reducing power, followed by red algae *H. musciformis and G. latifolium.* The results were similar to that obtained in the present investigation.

Table 3: Antioxidant Activity by Reducing Power Assay

Concentration (µg/ml)	Absorbance value		
	Reducing power assay		Standard (Ascorbic acid)
	C. vulgaris AS-13	*C. pyrenoidosa* AS-6	
62	0.114±0.002	0.098±0.003	0.248±0.005
125	0.133±0.003	0.118±0.002	0.372±0.007

Table 3: Contd.,			
250	0.136±0.003	0.124±0.001	0.456±0.008
500	0.182±0.004	0.156±0.003	0.474±0.010
1000	0.268±0.004	0.189±0.003	0.710±0.123

Results are expressed as the average of triplicate determination ± standard deviation

The Methanolic extract *Chlorella vulgaris* AS-13 was tested to the normal cell (WRL68), breast cancer cell (MCF7) and cancer cell lines (HepG2). The cytotoxicity of *Chlorella vulgaris* AS-13 with Methanol extract showed 50% inhibition (IC50) of cells with 92 µg/ml after 72 hours period of incubation for MCF7 when compared to HepG2 cell lines. There slightly indicates that there was some inhibition did not reach (IC50) on at 24 and 48 hour treatment period for both cancer cell lines. The HepG2 also showed the same effect after 72 hour treatment period (Table 4). Whereas there was a positive effect of methanolic extract *Chlorella pyrenoidosa* AS-6 on MCF7 at 72 hour period with 87 µg/mL. There was an indication that there was some inhibition but did not reach (IC50) at 24 and 48 hours (Table 5). The Cytotoxic assay of *Chlorella pyrenoidosa* AS-6 methanol extract on WRL68 indicates there was slightly inhibition at 48 and 72 hour but not reached 50% inhibition.

Table 4: Cytotoxic Assay of Methanolic Extract *Chlorella Vulgaris* AS-13 on Breast Cancer Cells (MCF7), Human Liver Cancer Cell Line (HepG2) and Normal Cells (24, 48 and 72 Treatment Period)

Cell lines	MCF7			WRL68			HepG2			WRL68		
Treatment periods (hours)	24	48	72	24	48	72	24	48	72	24	48	72
Effective conc^{n} (µg/mL)			92									
Occurrence of IC50	*	*	+	-	-	-	*	*	*	-	-	-

(+) indicates that there was 50% inhibition (IC50) of cells

(-) indicates that there was no inhibition of cells

(*) indicates that there was some inhibition but did not reach (IC50)

Table 5: Cytotoxic Assay of Methanolic Extract of *Chlorella Pyrenoidosa* AS-6 on Breast Cancer Cells (MCF7) and Normal Cells (24, 48 and 72 Treatment Period)

Cell Lines	MCF7			WRL68		
Treatment periods (hours)	24	48	72	24	48	72
Effective conc^{n} (µg/mL)			87			
Occurrence of IC50	*	*	*	-	*	*

(+) indicates that there was 50% inhibition (IC50) of cells
(-) indicates that there was no inhibition of cells
(*) indicates that there was some inhibition but did not reach (IC50)

In the present investigation *Chlorella vulgaris* AS-13 and *Chlorella pyrenoidosa* AS-6 were tested to the several cell lines such as breast cancer (MCF7), normal cell (WRL68) and human liver cancer cell line (HepG2). The inhibit proliferation of human breast cancer (MCF7) was better in Methanolic extract compared to the human liver cancer cell line (HepG2) with the same solvent. *Chlorella vulgaris* AS-13 extract inhibited the proliferation of MCF7 in a concentration-dependent manner, ranging from 0-200 µg/ml as shown by the cytotoxic assay. The HepG2 also showed the inhibition at 24, 48 and 72 treatment periods but not reached IC50 values. The Cytotoxic assay of *Chlorella pyrenoidosa* AS-6 methanolic extract shown better results on breast cancer cells (MCF7) compared to the HepG2 with 87 µg/mL. Whereas, the *Chlorella vulgaris* AS-13 methanol extract gave the small effect on both MCF7 and no effect at all for HepG2 cell lines. Most anticancer drugs exert their anticancer effects at the G1 or G2 stage to inhibit cell cycle progression

(16). Although *Chlorella vulgaris* AS-13 extract inhibited the growth of normal WRL 68 cells, this however, did not induce significant apoptosis in normal cells as shown in Table 4. In the present study, the dose-response experiments showed that Methanolic extract *Chlorella vulgaris* AS-13 generated larger inhibition on cytotoxicity to the MCF7 with low doses such as 92 μg/mL compared to the HepG2 cell lines. Whereas for the methanolic extracts *Chlorella pyrenoidosa* AS-6 also generated the nearby effect at 87 μg/mL to reach the 50% inhibition (IC50) of cells.

CONCLUSIONS

The present investigation revealed that *Chlorella vulgaris* AS-13 has the highest amount of phenol and flavonoid content when compared to *Chlorella pyrenoidosa* AS-6, the other tested algae. The methanolic extracts of *Chlorella vulgaris* AS-13 showed the maximum activity for Free radical scavenging assay i. e. DPPH and Phosphomolybdate assay. The Antioxidant activity by reducing power assay was also found to be maximum in methanolic extracts of *Chlorella vulgaris* AS-13 when compared to IC50 values of ascorbic acid, taken as standard. A methanol extract of *Chlorella vulgaris* AS-13 showed 50% inhibition (IC50) of cells with 92 μg/ml after 72 hours period incubation for MCF7 when compared to HepG2 cell lines. There was a positive effect of methanolic extract of *Chlorella pyrenoidosa* AS-6 on MCF7 at 72 hour period of with 87 μg/mL. Hence by the present study, it can be concluded that both of the microalgae in dietary supplements will be effective mediators in scavenging free radicals and act as antiapoptotic peptides for tumor cells.

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